

## DESCRIPTION

### ALKALINE PROTEASES

#### Technical Field

5           The present invention relates to alkaline proteases having high specific activity and strong oxidant resistance and as an enzyme to be added to a detergent, having excellent detergency.

#### 10   Background Art

          Proteases have conventionally been used in a variety of fields such as various detergents including laundry detergents, cosmetics, bath agents, food modifiers, and pharmaceuticals such as digestion aids and anti-  
15   inflammatory agents. Among them, proteases for detergents are industrially produced in the largest amount and have a great market scale. A number of proteases are now put on the market.

          In most cases, stains on clothes contain not only  
20   proteins but also plural components such as lipids and solid particles. There is accordingly a demand for detergents having detergency high enough to remove such actual complex stains. Finding, from such a viewpoint,

alkaline proteases capable of retaining caseinolytic activity even in the presence of a high concentration of fatty acids and exhibiting excellent detergency even if the stain is composed of not a simple protein component but plural components, for example, protein and lipid, and having a molecular weight of about 43,000, the present inventors applied a patent (WO99/18218) on them.

Alkaline proteases superior to the above-described ones in specific activity, oxidant resistance and detergency and usable for detergents of wide-ranging compositions have however been requested.

#### Disclosure of the Invention

The present inventors searched for such alkaline proteases mainly from enzyme variants. The above-described alkaline proteases are however utterly different in enzymological properties from serine proteases typified by subtilisin so that the modified site of subtilisin did not provide them with useful information. As a result of a further investigation, the present inventors have found that in order to obtain novel alkaline proteases having improved specific activity, stability against an oxidant and detergency while maintaining the properties of the

above-described alkaline proteases, they must have a specific amino acid residue at a predetermined position of their amino acid sequence.

In one aspect of the present invention, there is thus provided an alkaline protease wherein an amino acid residue at (a) position 84, (b) position 104, (c) position 256 or (d) position 369 of SEQ ID NO:1 or at a position corresponding thereto has been deleted or selected from:

at position (a): an arginine residue,  
at position (b): a proline residue,  
at position (c): an alanine, serine, glutamine, valine, leucine, asparagine, glutamic acid or aspartic acid residue, and  
at position (d): an aspartic acid residue.

In another aspect of the present invention, there is also provided an alkaline protease wherein an amino acid residue at (e) position 66 or 264, (f) position 57, each of 101 to 106, 136, 193 or 342, (g) position 46 or 205, (h) position 54, 119, 138, 148 or 195, (i) position 247, (j) position 124, (k) position 107 or (l) position 257 of SEQ ID NO:1, or at a position corresponding thereto has been deleted or selected from:

at position (e): a glutamine, aspartic acid, serine,

glutamic acid, alanine, threonine, leucine, methionine,  
cysteine, valine, glycine or isoleucine residue

at position (f): a lysine, serine, glutamine,  
phenylalanine, valine, arginine, tyrosine, leucine,

5 isoleucine, threonine, methionine, cysteine, tryptophan,  
aspartic acid, glutamic acid, histidine, proline or alanine  
residue,

at position (g): a tyrosine, tryptophan, alanine,  
asparagine, glutamic acid, threonine, valine, leucine,  
10 isoleucine, histidine, serine, lysine, glutamine,  
methionine or cysteine residue,

at position (h): a tryptophan, phenylalanine,  
alanine, asparagine, glutamic acid, threonine, valine,  
histidine, serine, lysine, glutamine, methionine, glycine,  
15 aspartic acid, proline, arginine or cysteine residue,

at position (i): a tryptophan, phenylalanine,  
alanine, asparagine, glutamic acid, threonine, valine,  
leucine, isoleucine, histidine, serine, glutamine,  
methionine or cysteine residue,

20 at position (j): an alanine or lysine residue,

at position (k): a lysine, arginine, alanine or  
serine residue, and

at position (l): a valine or isoleucine residue.

In a further aspect of the present invention, there are also provided a gene encoding the alkaline protease, a recombinant vector containing the gene and a transformant containing the vector.

5 In a still further aspect of the present invention, there is also provided a detergent composition containing the alkaline protease.

#### **Brief Description of the Drawings**

10 FIG. 1 is a diagram illustrating detergency of an alkaline protease variant; FIG. 2 is a diagram illustrating relative specific activity of each of alkaline protease variants; and FIG. 3 is a diagram illustrating relative residual activity of each of alkaline protease variants  
15 after treatment with an oxidant.

#### **Best Mode for Carrying Out the Invention**

As described above, in the alkaline proteases of the present invention, an amino acid residue at (a) position  
20 84, (b) position 104, (c) position 256 or (d) position 369 of SEQ ID NO:1 or at a position corresponding thereto has been deleted or selected from: at position (a): an arginine residue, at position (b): a proline residue, at position

(c): an alanine, serine, glutamine, valine, leucine, asparagine, glutamic acid or aspartic acid residue, and at position (d): an aspartic acid residue; or an amino acid residue at (e) position 66 or 264, (f) position 57, each of 5 101 to 106, 136, 193 or 342, (g) position 46 or 205, (h) position 54, 119, 138, 148 or 195, (i) position 247, (j) position 124, (k) position 107 or (l) position 257 of SEQ ID NO:1 or at a position corresponding thereto has been deleted or selected from: at position (e): a glutamine, 10 aspartic acid, serine, glutamic acid, alanine, threonine, leucine, methionine, cysteine, valine, glycine or isoleucine residue, at position (f): a lysine, serine, glutamine, phenylalanine, valine, arginine, tyrosine, leucine, isoleucine, threonine, methionine, cysteine, 15 tryptophan, aspartic acid, glutamic acid, histidine, proline or alanine residue, at position (g): a tyrosine, tryptophan, alanine, asparagine, glutamic acid, threonine, valine, leucine, isoleucine, histidine, serine, lysine, glutamine, methionine or cysteine residue, at position (h): 20 a tryptophan, phenylalanine, alanine, asparagine, glutamic acid, threonine, valine, histidine, serine, lysine, glutamine, methionine, glycine, aspartic acid, proline, arginine or cysteine residue, at position (i): a

tryptophan, phenylalanine, alanine, asparagine, glutamic acid, threonine, valine, leucine, isoleucine, histidine, serine, glutamine, methionine or cysteine residue, at position (j): an alanine or lysine residue, at position  
5 (k): a lysine, arginine, alanine or serine residue, and at position (l): a valine or isoleucine residue.

Specifically, the alkaline proteases according to the present invention mean alkaline proteases having an amino acid sequence represented by SEQ ID NO:1 wherein the amino acid residue at a position selected from the above-  
10 described (a) to (d) and (e) to (l) has been deleted or predetermined, or another alkaline protease wherein the amino acid residue at a position corresponding thereto has been deleted or predetermined. They may be wild type  
15 enzymes, wild type variants or artificial variants.

The "another alkaline protease" may be a wild type enzyme or a wild type variant. That having oxidant resistance and having a molecular weight, as determined by SDS-PAGE, of  $43,000 \pm 2,000$  is preferred, of which that  
20 having an amino acid sequence showing at least 60% homology with the amino acid sequence of SEQ ID NO:1 is more preferred. Particularly preferred is that having an amino acid sequence showing at least 60% homology with the amino

acid sequence of SEQ ID NO:1, having oxidant resistance, works on the alkaline side (pH 8 or greater), is stable with at least 80% residual activity when treated at pH 10 for 10 minutes even at 50°C, is inhibited by diisopropyl fluorophosphate (DFP) and phenylmethane sulfonyl fluoride (PMSF) and has a molecular weight, as determined by SDS-PAGE, of 43,000 ± 2,000. The term "having oxidant resistance" as used herein means that it has at least 50% of residual activity (synthetic substrate assay) when treated in a 50 mM hydrogen peroxide solution (containing 5 mM calcium chloride) at pH 10 (a 20 mM Britton-Robinson buffer) at 20°C for 20 minutes.

Examples of the "alkaline protease having an amino acid sequence represented by SEQ ID NO:1" include KP43 [derived from *Bacillus* sp. strain KSM-KP43 (FERM BP-6532), WO99/18218], while those of the "alkaline protease having an amino acid sequence showing at least 60% homology with the amino acid sequence of SEQ ID NO:1" include protease KP9860 having an amino acid sequence represented by SEQ ID NO:2 [derived from *Bacillus* sp. strain KSM-KP9860 (FERM BP-6534), WO99/18218], Protease E-1 having an amino acid sequence represented by SEQ ID NO:3 [derived from *Bacillus* sp. strain No. D-6 (FERM P-1592), JP740710], Protease Ya



having an amino acid sequence represented by SEQ ID NO:4

[derived from *Bacillus* sp. strain Y (FERM BP-1029),

JP861210], Protease SD521 having an amino acid sequence

represented by SEQ ID NO:5 [derived from *Bacillus* sp.

5 strain SD-521 (FERM BP-11162), JP910821], Protease A-1

having an amino acid sequence represented by SEQ ID NO:6

(derived from NCIB12289, WO8801293), and Protease A-2

having an amino acid sequence represented by SEQ ID NO:7

(derived from NCIB12513, WO8801293). Of these, the amino

10 acid sequences selected from SEQ ID NOS. 2 to 7 or alkaline

proteases showing at least 80%, more preferably at least

90%, especially at least 95% homology therewith are

preferred.

The homology of an amino acid sequence is calculated

15 by Lipman-Pearson's method (Science, 227, 1435(1985)).

The "amino acid residue at a corresponding position"

can be identified by comparing amino acid sequences by

using known algorithm, for example, that of Lipman-Pearson.

The position of the "amino acid residue at a corresponding

20 position" in the sequence of each protease can be

determined by aligning the amino acid sequence of the

protease in such a manner. It is presumed that the

corresponding position exists at the three-dimensionally

same position in the amino acid sequence of SEQ ID NO:1 and the amino acid residue existing at the same position brings about similar effects for a specific function of the protease.

5 Described specifically, (a) the amino acid residue at position 84 of SEQ ID NO:1 is a lysine residue. By employing the above-described method, the amino acid residue at the position corresponding thereto can be identified as the lysine residue at position 83 of SEQ ID  
10 NO:3. This amino acid residue is preferably arginine.

(b) Although the amino acid residue at position 104 of SEQ ID NO:1 is a leucine residue, this amino acid residue or an amino acid residue corresponding thereto is preferably a proline residue.

15 (c) Although the amino acid residue at position 256 of SEQ ID NO:1 is a methionine residue, particularly preferred as this amino acid residue is an alanine, serine, glutamine, valine, leucine, asparagine, glutamic acid or aspartic acid residue.

20 (d) Although the amino acid residue at position 369 of SEQ ID NO:1 is an aspartic acid residue, this amino acid residue or amino acid residue corresponding thereto is preferably an asparagine residue.

(e) Although the amino acid residue at position 66 or 264 of SEQ ID NO:1 is an asparagine residue, this amino acid residue is preferably a glutamine, aspartic acid, serine, glutamic acid, alanine, threonine, leucine, methionine, cysteine, valine, glycine or isoleucine residue, with an aspartic acid, serine or glutamic acid residue being particularly preferred. More preferred is the case wherein the amino acid residue at position 66 is an aspartic acid residue and that at position 264 is a serine residue.

(f) Although the amino acid residue at each of positions 57, 101 to 106, 136, 193 and 342 of SEQ ID NO:1 is a glycine residue, this amino acid residue is preferably a lysine, serine, glutamine, phenylalanine, valine, arginine, tyrosine, leucine, isoleucine, threonine, methionine, cysteine, tryptophan, aspartic acid, glutamic acid, histidine, proline or alanine residue. Particularly preferred is the case wherein the amino acid residue at position 57, 136, 193 or 342 is an alanine residue, or that at position 103 is an arginine residue.

(g) Although the amino acid residue at position 46 or 205 of SEQ ID NO:1 is a phenylalanine residue, this amino acid residue is preferably a tyrosine, tryptophan, alanine,

asparagine, glutamic acid, threonine, valine, leucine,  
isoleucine, histidine, serine, lysine, glutamine,  
methionine or cysteine residue. Particularly preferred is  
the case wherein the amino acid residue at position 46 is a  
5 leucine residue.

(h) Although the amino acid residue at position 54,  
119, 138, 148 or 195 of SEQ ID NO:1 is a tyrosine residue,  
this amino acid residue is preferably a tryptophan,  
phenylalanine, alanine, asparagine, glutamic acid,  
10 threonine, valine, histidine, serine, glutamine,  
methionine, glycine, aspartic acid, proline, lysine,  
arginine or cysteine residue. Particularly preferred is  
the case wherein the amino acid residue at position 195 is  
an alanine, aspartic acid, glutamic acid, glutamine,  
15 valine, tryptophan, glycine, lysine, threonine, methionine,  
cysteine, phenylalanine, proline, serine, arginine,  
asparagine or histidine residue.

(i) Although the amino acid residue at position 247  
of SEQ ID NO:1 is a lysine residue, this amino acid residue  
20 is preferably a tryptophan, phenylalanine, alanine,  
asparagine, glutamic acid, threonine, valine, leucine,  
isoleucine, histidine, serine, glutamine, methionine or  
cysteine residue. As the amino acid residue at position

247, an arginine or threonine residue is particularly preferred.

(j) Although the amino acid residue at position 124 of SEQ ID NO:1 is an arginine residue, this amino acid residue is preferably an alanine or lysine residue.

(k) Although the amino acid residue at position 107 of SEQ ID NO:1 is a leucine residue, this amino acid residue is preferably a lysine, arginine, alanine or serine residue, with a lysine residue being particularly preferred.

(l) Although the amino acid residue at position 257 of SEQ ID NO:1 is an alanine residue, this amino acid residue is preferably a valine or isoleucine residue, with a valine residue being particularly preferred.

With regards to "another alkali protease" which is preferred among the above-exemplified ones, positions corresponding to (a) to (d) and (e) to (l) of the amino acid sequence (SEQ ID NO:1) of Protease KP43 and specific examples of an amino acid residue are shown below (Table 1-a, Table 1-b).

Table 1-a

Position	Proteases						
	TS43 SEQ ID NO:1	9860 SEQ ID NO:2	E-1 SEQ ID NO:3	Ya SEQ ID NO:4	SD-521 SEQ ID NO:5	A-1 SEQ ID NO:6	A-2 SEQ ID NO:7
(a)	84Lys	84Lys	83Lys	83Lys	83Lys	84Lys	83Lys
(b)	104Leu	104Leu	103Leu	103Leu	103Leu	104Leu	103Leu
(c)	256Met	256Met	255Met	255Met	255Met	256Met	255Met
(d)	369Asp	369Asp	368Asp	368Asp	368Asp	369Asp	368Asp

Table 1-b

Position	Proteases						
	TS43 SEQ ID NO:1	9860 SEQ ID NO:2	E-1 SEQ ID NO:3	Ya SEQ ID NO:4	SD-521 SEQ ID NO:5	A-1 SEQ ID NO:6	A-2 SEQ ID NO:7
(e)	66Asn 264Asn	66Asn 264Asn	66Asn 263Asn	66Asn 263Asn	66Asn 263Asn	66Asn 264Asn	66Asn 263Asn
(f)	57Gly	57Gly	56Gly	56Gly	56Gly	57Gly	56Gly
	101Gly	101Ser	100Ser	100Ser	100Ser	101Asn	100Gly
	102Gly	102Gly	101Gly	101Gly	101Gly	102Gly	101Gly
	103Gly	103Gly	102Gly	102Gly	102Gly	103Gly	102Gly
	105Gly	105Gly	104Gly	104Gly	104Gly	105Gly	104Gly
	106Gly	106Gly	105Gly	105Gly	105Gly	106Gly	105Gly
	136Gly	136Gly	135Gly	135Gly	135Gly	136Gly	135Gly
	193Gly	193Gly	192Gly	192Gly	192Gly	193Gly	192Gly
(g)	342Gly	342Gly	341Gly	341Gly	341Gly	342Gly	341Gly
	46Phe 205Phe	46Phe 205Phe	46Phe 204Phe	46Phe 204Phe	46Phe 204Phe	46Phe 205Phe	46Phe 204Phe
(h)	195Tyr	195Tyr	194Ile	194Ile	194Leu	195Tyr	194Tyr
(i)	247Lys	247Lys	246Lys	246Lys	246Lys	247Lys	246Lys
(j)	124Arg	124Arg	123Arg	123Arg	123Arg	124Arg	123Arg
(k)	107Leu	107Leu	106Leu	106Leu	106Leu	107Leu	106Leu
(l)	257Ala	257Ala	256Ala	256Ala	256Ala	257Ala	256Ala

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In the alkaline proteases of the present invention, deletion of an amino acid residue or selection in (a) to (d) or (e) to (l) may be conducted at two or more positions simultaneously.

When the alkaline protease of the present invention is a variant, the "protease having an amino acid sequence represented by SEQ ID NO:1" or the above-exemplified "another alkaline protease" serves as an alkaline protease prior to mutation (which may be called "parent alkaline protease"). By introducing mutation to a desired site of this parent alkaline protease, the alkaline protease of the present invention is available. For example, it is available by deleting or substituting, with another amino acid residue, the amino acid residue at a position selected from the above-described (a) to (d) and (e) to (l) of the amino acid sequence of SEQ ID NO:1 of Protease KP43 or at the corresponding position of the amino acid sequence of another alkaline protease, more specifically, amino acid sequence represented by SEQ ID NOS:2 to 7.

The alkaline protease of the present invention can be obtained, for example, by introducing mutation to a cloned gene encoding a parent alkaline protease, transforming a proper host by using the resulting mutated gene and then culturing the recombinant host. Cloning of the gene for encoding a parent alkaline protease may be carried out using an ordinary gene recombination technique, for example, in accordance with the process as described in

WO99/18218, JP901128 or WO98/56927.

For mutation of a gene encoding a parent alkaline protease, either one of random mutation or site-specific mutation which is prevalent now can be adopted. More specifically, mutation can be effected using, for example, "Site-Directed Mutagenesis System Mutan-Super Express Kit" of Takara Shuzo Co., Ltd. By using recombinant PCR (polymerase chain reaction) as described in "PCR protocols" (Academic Press, New York, 1990), a desired sequence of a gene can be replaced with a sequence of another gene corresponding to the desired sequence.

For production of the protease variant of the present invention by using the resulting mutated gene, usable is, for example, the following process. A DNA encoding the protease variant of the present invention is stably amplified by linking the mutated gene with a DNA vector capable of amplifying it stably or by introducing the mutated gene onto a chromosomal DNA capable of maintaining it stably and then, the gene is introduced into a host permitting stable and efficient expression of the gene, whereby the variant protease is produced. Hosts satisfying the above-described conditions include microorganisms belonging to *Bacillus* sp., *Escherichia coli*, mold, yeast



and *Actinomyces*.

The alkaline protease of the present invention thus obtained has stable protease activity in an alkaline region, is free from the inhibition of caseinolytic activity by higher fatty acids, and has a molecular weight, as determined by SDS-PAGE,  $43,000 \pm 2,000$ . For example, the protease variant available from, as a parent strain, the protease having an amino acid sequence of SEQ ID NO:1 has the below-described physicochemical properties.

(i) Acting pH range

It acts in a wide pH range of from 4 to 13 and exhibits at least 80% of the optimum pH active value at pH 6 to 12.

(ii) Stable pH range

It is stable within a pH range of 6 to 11 under the treating conditions at 40°C for 30 minutes.

(iii) Influence of fatty acids

Its caseinolytic activity is not inhibited by oleic acid.

Such proteases of the present invention have excellent specific activity, oxidant resistance and detergency and are therefore useful as an enzyme to be incorporated in various detergent compositions.

Particularly, the proteases wherein the amino acid residue at position (a) to (d) of SEQ ID NO:1 or at a position corresponding thereto has been deleted or specified are superior in detergency. Among them, those having, as the amino acid residue at (c) position 256 or at a position corresponding thereto, an alanine, serine, glutamine, valine, leucine, asparagine, glutamic acid or aspartic acid residue have both high specific activity and strong oxidant resistance. The proteases wherein the amino acid residue at position (e) to (l) of SEQ ID NO:1 or at a position corresponding thereto has been deleted or specified have particularly excellent specific activity.

The above-described protease may be added to the detergent composition of the present invention in an amount sufficient to permit exhibition of its activity. Although 0.1 to 5000 P.U. can be added per 1 kg of the detergent composition, 1000 P.U. or less, preferably 500 P.U. is added in consideration of economy.

To the detergent composition of the present invention, various enzymes can be used in combination with the alkaline protease of the present invention. Examples include hydrolases, oxidases, reductases, transferases, lyases, isomerases, ligases and synthetases. Of these,

proteases, cellulases, lipases, keratinases, esterases,  
cutinases, amylases, pullulanases, pectinases, mannases,  
glucosidases, glucanases, cholesterol oxidases,  
peroxidases, laccases and proteases other than the alkaline  
5 protease used in the present invention.

Proteases include commercially available Alcalase,  
Espersase, Savinase and Everlase (each, product of Novo  
Nordisk), Properase and Purafect (each, product of Genencor  
International Inc.), and KAP (Kao Corp). Cellulases  
10 include Cellzyme and Carezyme (each, product of Novo  
Nordisk), KAC (Kao Corp.) and alkaline cellulase produced  
by *Bacillus* sp. strain KSM-S237 as described in Japanese  
Patent Application Laid-Open No. Hei 10-313859. Amylases  
include Termamyl and Duramyl (each, product of Novo  
15 Nordisk), Purastar (Genencor International Inc.), and KAM  
(Kao Corp.). Lipases include Lipolase and Liporase Ultra  
(each, product of Novo Nordisk). The above-exemplified  
enzyme may be incorporated in an amount of 0.001 to 10%,  
preferably 0.03 to 5%.

20 A surfactant may be incorporated in an amount of 0.5  
to 60 wt.% (which will hereinafter be called "%", simply)  
in the detergent composition. To a powdery detergent  
composition and a liquid detergent composition, addition of

10 to 45% and 20 to 50% are preferred, respectively. When the detergent composition of the present invention is a bleaching detergent or automatic dishwasher detergent, the surfactant may usually be added in an amount of 1 to 10%, preferably 1 to 5%.

A divalent metal ion scavenger may be added in an amount of 0.01 to 50%, with 5 to 40% being preferred.

An alkali agent and inorganic salt may be added in an amount of 0.01 to 80%, preferably 1 to 40%.

An antisoil redeposition agent may be added in an amount of 0.001 to 10%, preferably 1 to 5%.

A bleaching agent (ex. hydrogen peroxide or percarbonate) is added preferably in an amount of 1 to 10%. Upon use of the bleaching agent, 0.01 to 10% of a bleaching activator can be added.

As a fluorescent brightener, biphenyl type ones (such as "Tinopal CBS-X") and stilbene type ones (such as DM fluorescent dye) can be used. It is added preferably in an amount of 0.001 to 2%.

The detergent composition of the present invention can be prepared in a conventional manner by using the alkaline protease obtained by the above-described process and the above-described known detergent components in

combination. The detergent form can be selected according to the using purpose. Examples include liquid, powder and granule.

When the alkaline protease of the present invention is added to a powdery detergent composition, it is preferred to prepare detergent particles in advance and then mix therein the alkaline protease granules in accordance with the process as described in Japanese Patent Application Laid-Open No. Sho 62-25790 in order to avoid the contact of workers or end users with the enzyme upon preparation or use of the detergent or to prevent heat-induced deactivation or decomposition of the enzyme.

The detergent composition of the present invention thus available is usable as a laundry detergent, bleaching detergent, automatic dishwasher detergent, pipe cleaner and artificial tooth cleaner. Use as a laundry detergent, bleaching detergent or automatic dishwasher detergent is particularly preferred.

#### Example 1

Mutation was introduced at random into a protease structural gene of about 2.0 kb including a termination codon by the following manner. First, PCR was conducted

using a primer capable of amplifying this 2.0kb. A PCR master mix contained 5 ng of a template DNA, 20 pmol of a phosphorylated primer, 20 nmol of each dNTP, 1 imol of Tris/HCl (pH 8.3), 5 imol of KCl, 0.15 imol of MgCl<sub>2</sub> and 2.5U TaqDNA polymerase, and its total amount was adjusted to 100 iL. After modification of the template by allowing it to stand at 94°C for 5 minutes, PCR was performed for 30 cycles, each cycle consisting of treatment at 94°C for 1 min, at 55°C for 1 min and at 72°C for 1.5 min. The PCR product was purified by "PCR product purification Kit" (product of Boeringer Mannheim), followed by elution in 100 iL of sterile water. With 1 iL of the eluate, second PCR was conducted under conditions similar to those of the first PCR except for the template DNA. After completion of the second PCR, the PCR product was purified in a similar manner to the first PCR, followed by elution in 100 iL of sterile water.

The amplified DNA fragment was integrated in a vector by polymerase reaction using "LATaq" produced by Takara Shuzo Co., Ltd. Described specifically, after addition of 5 iL of a buffer for LATaq (a 10-fold concentrate), 8 iL of a dNTP solution and 0.5 iL of LATaq DNA polymerase, and as a template, 20 ng of plasmid pHA64TS (having a protease

structural gene linked with an expression vector pHA64) to 35  $\mu$ L of the purified eluate, the total amount was adjusted to 50  $\mu$ L. PCR reaction of the resulting liquid was carried out for 30 cycles, each consisting of treatment at 94°C for 1 min, 55°C for 1 min and 72°C for 4 min. By the subsequent ethanol precipitation, the PCR product was collected. This PCR product had a shape of a plasmid having a nick at the 5' prime end of the primer. Ligase reaction by T4 ligase (product of Takara Shuzo Co., Ltd.) was conducted to link this nick portion.

By using 10  $\mu$ L of this ligase reaction mixture, transformation of the *Bacillus subtilis* strain ISW1214 was conducted, whereby about  $4 \times 10^5$  transformants were obtained. The resulting transformants of the strain ISW1214 were cultured on a skin-milk-containing medium (containing 1% skim milk, 1% bactotrypton, 1% sodium chloride, 0.5% yeast extract, 1.5% agar and 7.5  $\mu$ g/ml of tetracycline) and halo formation, which was presumed to reflect the protease secretion amount, was observed.

## Example 2: Purification of an enzyme

The protease active fraction was prepared in the following manner. The transformants obtained in Example 1 was cultured at 30°C for 60 hours on a medium A (3%

polypeptone S (product of Nippon Pharmaceutical), 0.5% yeast extract, 1% fish meat extract (product of Wako Pure Chemical Industries, Ltd.), 0.15% dipotassium phosphate, 0.02% magnesium sulfate 7 hydrate, 4% maltose and 7.5  $\mu$ g/mL of tetracycline). The supernatant of the thus-obtained cultured medium was added with ammonium sulfate to give 90% saturation, whereby salting-out of protein was caused. The sample obtained by salting-out was dissolved in a 10 mM tris HCl buffer (pH 7.5) containing 2 mM of calcium chloride. The resulting solution was dialyzed overnight against the same buffer by using a dialysis membrane. The fraction in the dialysis membrane was applied to DEAE Bio-Gel A (product of Bio-Rad Laboratories) equilibrated with a 10 mM tris HCl buffer (pH 7.5) containing 2 mM calcium chloride to collect the protease active fraction not adsorbed to the ion-exchanger. This active fraction was applied further to "SP-Toyopearl 550W" (product of Tosoh Corp.) equilibrated with the same buffer, followed by elution with a 0 to 50 mM sodium chloride solution, whereby a protease active fraction was obtained. The resulting fraction was analyzed by SDS-PAGE electrophoresis to confirm that the protease was obtained as substantially uniform protein. The protein concentration was measured in



accordance with the method of Lowry, et al. (J. Biol. Chem. 193, 265-275(1981)) by using bovine serum albumin (product of Bio-Rad Laboratories) as a standard.

### Example 3: Measuring method of protease activity

#### 5 (1) Synthetic substrate assay

A decomposition rate was measured using a synthetic peptide made of Glt-Ala-Ala-Pro-Leu(A-A-P-L) as a substrate. Described specifically, a 50 mM borate/KCl buffer (pH 10.5) containing each enzyme to be evaluated and 10 3 mM of Glt-A-A-P-L-pNA (product of Peptide Institute, Inc) was kept at 30°C for 10 minutes and then, an absorbance at 420 nm was periodically measured. The peptide hydrolyzing activity was determined from an increasing ratio of the absorbance at 420 nm per unit hour. The protein was 15 determined using a protein assay kit of Bio-Rad Laboratories.

#### (2) Natural substrate assay

After 1.0 mL of a 50 mM borate buffer (pH 10) containing 1% (w/v) of casein was kept at 30°C for 5 20 minutes, 0.1 mL of an enzyme solution was added and reaction was conducted for 15 minutes. To the reaction mixture, 2.0 mL of a reaction-stopping solution (0.11M trichloroacetic acid - 0.22M sodium acetate - 0.33M acetic

acid) was added. The resulting mixture was allowed to stand at room temperature for 30 minutes and the filtered. The acid soluble protein in the filtrate was quantitatively determined by the modified method of Lowery, et al.

5 Described specifically, after addition of 2.5 mL of an alkaline copper solution [1% potassium sodium tartrate : 1% copper sulfate : 1% sodium carbonate = 1:1:100] to the filtrate, the resulting solution was allowed to stand at room temperature for 10 minutes. Then, 0.25 mL of a  
10 diluted phenol solution (a phenol reagent (product of Kanto Kagaku) diluted 2-fold with ion exchange water) was added. After the resulting mixture was kept at 30°C for 30 minutes, absorbance at 660 nm was measured. One enzyme unit was designated as a quantity of the enzyme for  
15 liberating the acid soluble protein hydrolysis product corresponding to 1 mmol of tyrosine for 1 min in the above-described reaction.

#### Example 4

##### (1) Preparation of granular detergent

20 Detergency of the detergent as described in Example 3 of W099/29830 was evaluated. Described specifically, 465 kg of water was poured in a mixing tank of 1 m<sup>3</sup> equipped with a stirring blade. After its water temperature reached

55°C, 48 kg of a 50% (w/v) aqueous solution of sodium dodecylbenzenesulfonate and 135 kg of a 40% (w/v) aqueous solution of sodium polyacrylate were added. After stirring for 15 minutes, 120 kg of sodium carbonate, 60 kg of sodium sulfate, 9 kg of sodium sulfite and 3 kg of a fluorescent dye were added. After stirring for further 15 minutes, 300 kg of zeolite was added. The mixture was stirred for 30 minutes to yield a uniform slurry (the slurry had a water content of 50 wt.%). By spraying this slurry from a pressure spraying nozzle disposed in the vicinity of the top of a spray drying tower, base granules were obtained (a high temperature gas was fed to the spray drying tower at 225°C from the tower bottom and discharged from the tower top at 105°C).

Then, 15 parts by weight of a nonionic surfactant, 15 parts by weight of a 50 wt.% aqueous solution of sodium dodecylbenzenesulfonate and 1 part by weight of polyethylene glycol were mixed under heating to 70°C, whereby a mixture was obtained. In a Loedige mixer (product of Matsuzaka Giken Co., Ltd., capacity: 20L, equipped with a jacket), 100 parts by weight of the base granules obtained above were charged and stirring by a main shaft (150 rpm) and chopper (4000 rpm) was started. Warm

water of 75°C was caused to flow in the jacket at 10 L/min, the mixture was charged therein in 3 minutes, and then stirring was conducted for 5 minutes. The surface of the detergent particles were covered with 10 parts by weight of crystalline aluminosilicate, whereby the final product of the granular detergent was obtained.

[Raw materials used]

Aqueous solution of sodium dodecylbenzenesulfonate:

"Neopelex F65" (product of Kao Corp.)

Nonionic surfactant: "Emulgen 108KM" (product of Kao) added with 8.5 moles, on average, of ethylene oxide

Aqueous solution of sodium polyacrylate: having an average molecular weight of 10000 (prepared in accordance with the process as described in Example of Japanese Patent Publication No. Hei 2-24283)

Sodium carbonate: dense ash (product of Central Glass Co., Ltd.)

Zeolite: "Zeolite 4A" having an average particle size of 3.5  $\mu$ m (product of Tosoh Corp)

Polyethylene glycol: "K-PEG6000" (average molecular weight of 8500, product of Kao Corp.)

Fluorescent dye: "Tinopal CBS-X" (product of Ciba Geigy)

## (2) Preparation of granulated protease

From the alkaline proteases of the present invention and a purified preparation of a parent alkaline protease, granulated protease was prepared based on the process as described in Japanese Patent Application Laid-Open No. Sho 62-257990 (6P.U./g)

## (3) Measurement of detergency

In 1L of an aqueous calcium solution (71.2 mg calcium carbonate/1L) adjusted to 20°C, 0.67 g of each of the detergent compositions as shown in Table 2 was dissolved. With the resulting solution, a test cloth ("EMPA117" (prepared by Swiss Federal Laboratories for Materials Testing and Research, blood/milk/carbon) cut into a piece of 6 × 6 cm was washed using a Terg-O-tometer (product of Ueshima Seisakusho) at 20°C and 100 rpm for 10 minutes. After rinsing and drying, the brightness was measured using a spectrophotometer ("CM3500d", product of MINOLTA). A detergency was calculated based on the below-described equation. The results are shown in Table 2.

$$\text{Detergency (\%)} = \frac{\text{Brightness of the test fabric after washing} - \text{that before washing}}{\text{Brightness of the test fabric before soiling} - \text{that before washing}} \times 100$$

Measuring results of the detergency of the protease variants obtained in Example 1 are shown in FIG. 1. The

alkaline protease variants of the present invention each exhibited superior detergency to wild type enzymes to which mutation had not been introduced.

Table 2

			Invention products					Comparative products	
			1	2	3	4	5	1	2
Parts by weight	Granulated alkaline proteases of this invention	K84R L104P M256S M256A D369N	0.5	0.5	0.5	0.5	0.5		
	Granulated parent alkaline protease							0.5	
	Granular detergent		99.5						100
Detergency (%)			38	38	36	36	34	31	23

#### Example 5

Measuring results, in accordance with the synthetic substrate assay or natural substrate assay, of protease activity of the protease variants obtained in Example 1

(the proteases modified at 195-position and 256-position amino acid residues, respectively was measured by the latter assay, while the other proteases were measured by the former assay) are shown in FIG. 2. The alkaline protease variants of the present invention exhibited high specific activity.

#### Example 6

In 2 mL of a 100 mM borate buffer (pH 10.5)

containing 3% of aqueous hydrogen peroxide, a 50 il portion of each of the protease variants obtained by purification in Example 1 was added. The resulting mixture was allowed to stand at 30°C for 30 minutes. After addition of an adequate amount of catalase (product of Boehringer Mannheim) to remove excess hydrogen peroxide, the residual protease activity was measured by the synthetic substrate assay. In FIG. 3, the residual activity after treatment with aqueous hydrogen peroxide is shown relative to the activity before treatment set at 100%.

The alkaline protease variants of the present invention exhibited higher oxidant resistance than the parent alkaline protease.

### **Industrial Applicability**

The present invention makes it possible to provide alkaline proteases having activity even under a high concentration of fatty acids, having high specific activity, oxidant resistance and detergency, and being useful as an enzyme to be incorporated in a detergent.